From The Department of Biosciences
Karolinska Institutet, Stockholm, Sweden

Histone deacetylases and their co-regulators in *Schizosaccharomyces pombe*

Rebecca Ann Silverstein

Karolinska Institutet

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Nothing is more useful than silence.

Menander of Athens (342 – 292 BC)

ABSTRACT

The DNA in every eukaryotic cell is wrapped around eight core histones to form the nucleosome. Therefore all events that involve DNA must also involve chromatin and nucleosomes. By regulating chromatin structure the cell can regulate the reactivity of the DNA. One of the most common ways of altering nucleosomes is the acetylation of lysine residues. Two enzymes are required to maintain the correct equilibrium for optimal cell growth: histone acetyltransferases (HATs) and histone deacetyltransferases (HDACs). In general, histone hypoacetylation is correlated with transcriptional inactivation, while hyperacetylation is correlated with active gene transcription.

In *Schizosaccharomyces pombe,* mating type loci are silenced. Deletion of HDAC Hos2 had previously been shown to slightly increase silencing at the mating type locus. To assess whether any other HDAC was necessary for mating type silencing, cells were treated with HDAC poison Trichostatin A (TSA). TSA was found to cause a mild derepression of the mating type locus, indicating that another HDAC was responsible for silencing in this region. The RNA interference nuclease Dcr1 was later identified, and showed to degrade double stranded RNA into small nucleotide fragments. Deletion of *dcr1* caused chromosome segregation defects and derepression of centromeric silencing.

Rpd3 in *S. cerevisiae* is recruited to genomic targets by interacting with co-regulator Sin3. *S. pombe* has three Sin3 homologs. Pst1 interacts with the HDAC Clr6, and like Clr6 is an essential gene, mutants of which display chromosome mis-segregation and derepression of centromeric silencing. Pst1 was required for centromeric cohesion, and localized to centromeres in late S phase. Thus a co-repressor paradigm could be applied to centromere silencing as well. A comparative characterization of HDACs in *S. pombe* showed that the HDACs had different localizations and histone specificities.

The comparison of HDACs was taken further with a genome wide expression analysis and histone density study of mutants. Results indicated that Clr6 was most often involved in promoter initiated gene repression, whereas Hos2 promoted the high expression of growth related genes by deacetylating H4K16ac in their coding regions. A class II HDAC, Clr3, was found to act cooperatively with Sir2 throughout the genome. Using a genomic approach to analyze Pst3, it was established that Clr6 and Pst3 could cooperate to negatively regulate genes by binding to their promoter regions. On the other hand, Pst3 was also involved in the up-regulation of ribosome biosynthesis genes, and could bind to the rDNA.

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LIST OF ABBREVIATIONS

Clr	Cryptic loci regulator
CtBP1	E1A C-terminal binding protein
DNA	Deoxyribonucleic acid
DOT1	Disruptor of telomeric silencing
ESET	ERG-associated protein with SET domain
EAF2	ELL-associated factor 2
GAGA	GAGA binding factor
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HMT	Histone methyltransferases
HOS	Hda1 similar
NAD	Nicotinamide adenine dinucleotide
N-CoR	Nuclear receptor co-repressor
NMR	Nuclear magnetic resonance
PABPC1	poly(A) binding protein C1
PAH	Paired amphipathic helix
PAZ	Polyubiquitin-associated zinc finger
Phd1	*S. pombe* histone deacetylase 1
Pst1	*S. pombe* Sin3 homolog 1
Pst2	*S. pombe* Sin3 homolog 2
Pst3	*S. pombe* Sin3 homolog 3
rDNA	DNA encoding ribosomal RNA
RNA	Ribonucleic acid
RPD3	Reduced potassium dependency
SAHA	HDAC inhibitor suberoylanilide hydroxamic acid
SDI	split SET/MYND domain-containing histone H3K36 specific methyltransferase
SET	Suppressor of variegation and enhancer of *Zeste* and *Trithorax*
SID	SIN3 Interaction Domain
SIN	Swi independent
SMRT	Silencing mediator for retinoic acid and thyroid hormone receptors
Smyd2	SET/MYND domain methyltransferase

Su(fu)	Suppressor of fu
SUMO	Small ubiquitin like modifier
TPX	HDAC inhibitor trapoxin
TSA	HDAC inhibitor trichostatin A
WD reapeat	Tryptophan Aspartate repeat
WICH	WSTF (Williams syndrome transcription factor)-Snf2h remodelling complex

1 GENERAL INTRODUCTION TO CHROMATIN

Deoxyribonucleic acid (DNA) stores the hereditary information required to regulate the nature and number of virtually all cellular molecules. Very long DNA molecules are organized into chromosomes in the eukaryotic nucleus. If all of the DNA in a human cell were stretched out into a single linear fiber, it is estimated that the total length would be over two meters. The cell, however, must package its DNA within a nucleus with a diameter of 10 microns. One key step in accomplishing this feat is the assembly of the DNA into chromatin.

1.1 THE NUCLEOSOME

The repetitive nucleoprotein complex, called the nucleosome, is the fundamental unit of chromatin. The core nucleosome particle is composed of 146 base pairs of DNA wrapped in two superhelical turns around a histone octamer. The core octamer is composed of two histone H3:H4 heterodimers that are assembled as tetramers in a new nucleosome with the help of chaperone proteins (Figures 1). Subsequently two H2A:H2B heterodimers are inserted into the core to form a complete nucleosome. Most histone:histone interactions in the nucleosome core are mediated via the central histone fold domains conserved in all core histones (3). The histone fold domain is composed of three α-helices separated by two short non-helical regions referred to as loops. The α-helices of the dimers clasp one another in a molecular handshake, resulting in extensive contact between the dimers (Figure 1). Importantly, the histone fold motif also contacts the inner surface of the DNA superhelix, primarily through the loop domains, and the first a-helix (77). The basic amino-terminal tails of the histones H3 and H2B have been portrayed in the crystal structure model as random coils protruding between the DNA superhelices encircling the nucleosome core approximately every 20 base pairs. These random coils have been predicted to interact with neighboring nucleosomes, or with the outer groove of the DNA. Although highly unstructured, histone H4 amino-terminal tails (H4K16 to H4R19) have been shown to interact with the H2A-H2B dimer of the adjacent nucleosome.

As the main architectural protein organizing eukaryotic genomes, histones are among the most evolutionarily conserved proteins in eukaryotes. Rudimentary histones even exist in Archaebacteria (99, 113). Additionally, the 102 amino acid sequence of histone H4 is identical between all mammals, and the sequence between plants and mammals only varies in two places. This has been attributed to selective evolutionary pressures towards forming extensive interfacial contacts between the core histones and within the octamer while maintaining contact with the inner surface of the DNA super-helix (3).

Compaction, however, is not the only function of the nucleosome. Even the Archaebacterial pre-histones seem to have a repressive influence on gene transcription. As organisms became more complex they had more DNA to package, but possible more importantly they also needed to evolve more refined systems of transcriptional control to support their need for both temporally and spatially coordinated gene expression. In principal, a system to manage DNA accessibility might then be said to be key to the development of multicellular organisms.

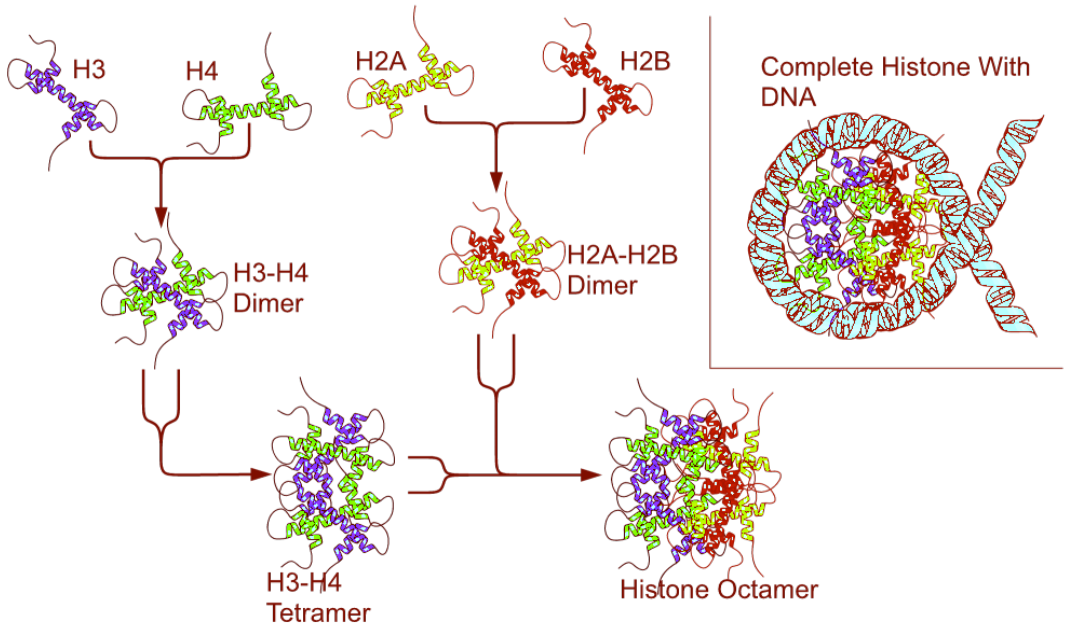

Figure 1. Nucleosome structure. Dimerization of histones H3::H4 and H2A::H2B occur via the histone fold domain. An H3::H4 tetramer is first inserted into the chromatin loop, and then is pinned in by two H2A::H2B dimers form a nucleosome particle.

Histone modification provides one layer of that accessibility system. Nucleosomes and the chromatin structure they establish interfere with all steps of gene transcription. By subtly changing the charges of the core histones, the whole nucleosome structure can be made more or less stable, and its interactions with DNA will be similarly affected. This in turn changes the nature of its internucleosomal contacts as well. Histone modification is also essential for establishing specialized chromatin structures central for genome stability.

1.2 HETEROCHROMATIN

Heterochromatin can be cytologically defined as the portion of chromatin that remains fully condensed throughout the cell cycle. Functionally, heterochromatin is described as those regions in a genome that are not amenable to transcription, replication, recombination, or repair. However, these ‘inert’ definitions do not denote the importance of heterochromatin in DNA replication, chromosome segregation or repair.

Heterochromatin can also be described in terms of its chromatin structure. In 1993, Jeppesen and Turner showed that constitutive and facultative heterochromatin in human and mouse cells were essentially free of histone acetylation when visualized with antibodies against acetylated histone H4 (52). We have since come to understand that the methylation of histone H3K9 is a conserved characteristic of heterochromatin from yeast to humans.

1.2.1 Centromeres

Centromeres appear under the microscope as highly condensed constrictions on metaphase chromosomes, where microtubules attached via the kinetochore serve to pull sister chromatids to opposite poles during anaphase. The underlying primary sequence of centromeres is highly repetitive, and with the exception of *S. cerevisiae*, contains no DNA elements sufficient to initiate centromere formation. Despite a lack of sequence homology, the structure of centromeres has been conserved through evolution.

In humans, centromeres are composed of 200 kb to 4 Mb of α-satellite DNA. The central region consists of ordered tangentially repeated 171 base pair sequences referred to as α-I satellite DNA (reviewed in 4). Flanking the inner region are regions containing divergent repetitive sequences and retrotransposons referred to as α-II satellite DNA. Under normal circumstances the centromere forms on the inner α-I satellite DNA sub-domain (reviewed in (147)). These inner and outer structures are accompanied by distinct functions that have been conserved through evolution, and are well characterized in *S. pombe.*

S. pombe has three centromeres that are approximately 35, 65, and 110 kb in length. The central core (*cnt*) domain consists of 4-7 kb of non-repetitive DNA and is flanked by centromere-unique inverted 'innermost' repeats (*imr*). The outer (*otr*) repeat elements *dg* (K) and *dh* (L) are highly homologous between the chromosomes and the *dg* element is also present at the mating type locus and in the telomeric regions (reviewed in (103)). The kinetochore, a large protein complex mediating microtubule attachment to the spindle pole body during chromosome segregation, assembles upon the *cnt* domain, whereas the heterochromatic nature of the *imr* and *otr* regions seem to be necessary for the proper centromere assembly (2, 65) at least in part because of its function in establishing sister chromatid cohesion (8).

Silencing mechanisms for *S. pombe* and human centromeres are also very similar insofar as they have been characterized in humans. In general, centromeres are hypoacetylated. More specifically, histone H3 must be deacetylated at lysine 9 before the methyltransferase Clr4 (SUV39H1) can gain access to it (6, 90). H3K9 methylation in turn is recognized by the chromodomain protein Swi6 (HP1 homolog), which in turn recruits cohesions necessary for proper kinetichore assembly. The heterochromatinization of this region is initiated by RNA inhibition (RNAi). In short, transcripts from the *otr* are converted to double stranded RNA by the RNA-dependent RNA polymerase, Rdp1. Those transcripts are then digested by Dicer (Dcr1) and incorporated into the RITS (RNA-induced initiation of transcriptional silencing) complex. RITS is composed of chromodomain protein Chp1, the siRNA binding protein Argonaute (Ago1) and Tas3. The complex is then targeted by the siRNA complementary to the centromere region, where it initiates the establishment of heterochromatin (reviewed in (29, 138)). The deletion of most of these components has been demonstrated to impair the function of the centromere and cause chromosome lagging: swi6 (30); clr4 (31); dcr1 (42, 105, 145); chp1 (24); ago1 (145); rdp1(145).

1.2.2 Telomeres

Telomeres are heterochromatic structures at the ends of chromosomes. The function of telomeres is in part to protect the chromosome from exonucleolytic attack, and preventing the cell's repair machinery from recognizing the chromosome ends as double strand breaks thus risking end-to-end fusions or cell cycle arrest. Telomeres also play a crucial role in chromosome dynamics in yeast (15, 57, 96). For example, in meiotic prophase, telomeres cluster and attach to the spindle pole body in a heterochromatin dependent fashion, forming what is known as a telomeric bouquet. These bouquets are conserved in most eukaryotic organisms, and are thought to facilitate chromosome pairing and homologous recombination (97, 114, 173).

1.2.3 rDNA

The rDNA is a specialized region of the chromosome that encodes the RNA molecules that will become part of the matured ribosome. The rDNA itself is localized at the core of the eukaryotic nucleolus, whereas the periphery of the nucleolus is populated by proteins necessary for processing and assembling the 40S and 60S ribosomal subunits. In *S. pombe* 100-120 tandem repeats of the rDNA gene encoding the 5.8S, 18S and 26S rRNA genes are located near the telomeres at each end of chromosome III. Like higher eukaryotes, the 5S rRNA genes are dispersed among the three chromosomes. Whereas the nucleolar rRNA genes are transcribed by RNA polymerase I, the 5S rRNA is transcribed by RNA polymerase III. This is used in part as a dosage regulator, but the heterochromatic environment is also needed to suppress recombination between the highly repetitive rDNA genes (reviewed in chapter 21.4 (27)).

1.2.4 Mating type in *S. Pombe*

In *S. pombe* the mating type locus is a very well characterized heterochromatic region. The mating type of a cell is determined by the expression from the mat1 locus. A region known as the *dg* (K) region lies between mat1 and the two other loci encoding silent mating type information: *mat2-P* and *mat3-M*. The K region is repressed for recombination, can repress transcription of polII genes inserted nearby, and has a repetitive sequence strikingly similar to the centromere repeats (40).

2 HISTONE MODIFICATIONS

In 1964, K. Murray began histone modification research when he established the presence of ε-methyllysine in calf thymus histones (88). We now understand that all four of the core histones are subjected to multiple covalent modifications that have dramatic effects for genome stability as well as gene regulation. Modifications of core histones include lysine acetylation, arginine or lysine methylation, serine or threonine phosphorylation, ubiquitination, sumoylation, glycosylation, and ADP-ribosylation. The modification map is further complicated by the multiple conformations some of the modifications can take. For instance, Arginine can be symmetrically or asymetrically dimethylated, or simply monomethylated. Methylation of lysine residues is also complicated by the addition of one, two or three methyl groups (reviewed in (83)).

Covalent post-translational modifications can be targeted to the histone fold domain to affect the nucleosome stability, as well as the N-terminal tails to modulate internucleosomal contacts and contacts with other chromatin proteins. Some histone modifications provide highly stable and heritable marks that define specialized regions of chromatin such as centromeres, telomeres, rDNA and mating type regions in yeasts. On the other hand, some histone marks are highly dynamic, enabling the adaptation of targeted chromatin regions to the rapidly changing needs of a living cell.

A recent comparative study of histone H3 and H4 modifications in *S. cerevisiae*, *Tetrahymena thermophila*, mouse and human revealed that the histones from unicellular organisms had more modifications correlated with transcriptional activation, whereas the mammalian histones tested had more repressive marks. As the authors point out, this probably reflects the fact that most of the genome in unicellular eukaryotes is potentially active, whereas 60% of the mammalian genome is permanently silenced. What is more, nearly all histone H3 lysines tend to be acetylated or methylated in each of the organisms tested; however, the H3 modification patterns were increasingly complex in correlation with the complexity of the organism. In contrast, histone H4 was found to carry fewer modifications than histone H3, and those modifications tended to be conserved between species. In light of these results, the authors argue that a universal histone code is unlikely, and warn against over-interpretation of modification results between organisms (38). That having been said, it seems there is a role to play for model organisms such as *S. pombe* in deciphering both the mechanisms of modification establishment and how the cell interprets the signal. And given the conservation in the histone H4 code, there is an obvious role for a simple model to understand why those modifications have been conserved.

The core of the histone particle is critically important for cell viability. In yeast, deletion of the tail domains of the H4 gene resulted in viable strains, albeit with globally disrupted chromatin structure. On the other hand, most deletions extending into the histone fold regions were lethal (61). In addition, histone H4 that has had its N-terminal tails removed by trypsination can still assemble into core nucleosomes in vitro (81). More recently it was demonstrated that tailless mutants of histones H3, H2A and H2B could also organize into nucleosomes in vitro as well, but that the tails of H4 were required for full compaction (25). In light of the histone models presented by Luger

et al, it seems reasonable to expect that nearly any deletion in the histone fold domain would interfere with the histone::histone interfaces, or with the nucleosome contacts with the DNA.

Although often neglected, there is plentiful evidence that covalent modifications within the histone folds are both common and important in vivo. By studying the Luger model closely, Cosgrove et. al identified 53 putative modification sites that could potentially influence the nucleoprotein interface (16). They predicted that these interface sites would affect nucleosome mobility and proposed a model whereby the primary function of histone modifications is to directly or indirectly control nucleosomal mobility. To support this model, they pointed out that all chromatin remodeling complexes to date have a mechanism for recognizing modified histone residues. The systematic characterization of histone modifications from calf thymus using peptide mass fingerprinting identified approximately 25 sites of histone modification within the histone fold regions (168).

One important modification within the core domain of histone H3 is the acetylation of H3K56, the last residue of the *a*-helix that connects the N-terminal histone H3 tail to the globular domain of H3 (reviewed by (100)). In *S. cerevisiae* H3K56 is essential. Nucleosomes assembled during S-phase are acetylated at H3K56 and the NAD^+ - dependant deacetylases HST3 and HST4 are responsible for the subsequent deacetylation of H3K56 in G2/M. In *hst3/hst4* mutants DNA lesions can be detected in the DNA after a single round of replication. After the DNA polymerase passes a second time, double stranded breaks begin to appear, an effect that can be repressed by H3K56R mutations that mimic the non-acetylated state. The fact that H3K56 could also be shown to be acetylated on calf thymus histones (168) suggest this modification could be conserved.

One conserved modification to the histone H3 globular region is the methylation of H3K79 exclusively governed by the non SET-domain histone metyltransferase (HMT) Dot1L in humans and Dot1p in *S. cerevisiae* (35) (134). Current models propose that Dot1p sets the ground conditions needed to distinguish euchromatin from heterochromatin in yeast. Dot1p is able to methylate 90% of the H3K79 in the genome that correlates with the euchromatin portion of the genome, whereas the silent heterochromatic regions such as telomeres and the mating type region lack this modification(134). It will be interesting to see whether this modification has a similar role in humans (Figure 2).

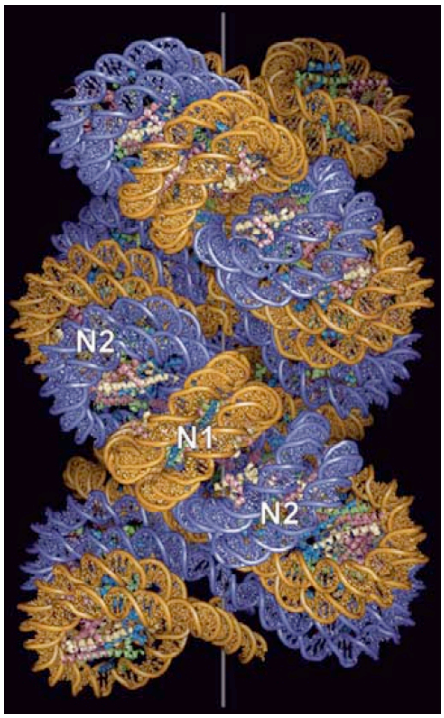

Figure 2. Tetranucleosome structure, as observed by *Schalch et al.*, Nature (2005) July 7; 436: 138-141

Cross-talk between histone modifications adds a level of complexity to interpreting histone modification patterns. H3K79 lies in the loop domain between α-helix 1 and 2

that Luger et al (77) predicted would be in a position to shelter modifiable residues at the H2AH2B interface. Indeed the ubiquitination of histone H2BK123 is a prerequisite for methylation of H3K79 by Dot1 in yeast (94, 129). The interdependence of modifications between histones has only begun to be explored. Considering the recently published tetranucleosome fine structure (Figure 3), interactions between nucleosomes will also be of great concern in understanding the final chromatin structure.

Although the basic N-terminal tails of the core histones are neither extensively involved in the histone fold interactions nor coordinate the DNA binding, they remain highly conserved. The N-terminal tails are key to nucleosome stability and the modulation of interactions between adjacent nucleosome particles, and therefore regulate the accessibility and stability of nucleosome arrays.

The methylation of H3K9 in combination with general hypoacetylation of the other residues is tightly linked to silencing. In S. pombe Clr4/Suv39 is responsible for the methylation of H3K9. This modification is in turn bound by the chromodomain of Swi6/HP1 to recruit silencing to the K region at *mat2 -3*, *imr1R*, *otr1R* but not the *cnt* (90).

Although methylation of H3K36 can be repressive if it is present in the promoter of a gene (70, 126), it has gained more attention for its role in the 5' of active coding regions where it suppresses aberrant transcriptional initiation in the wake of elongating RNA pol II (13, 54, 58). Using microarrays, Rao et al. determined that dormant genes become di-methylated at H3K36 upon activation (108). They also demonstrated that although nucleosomes are often cleared from the open reading frames of highly expressed genes, nucleosomes with the H3K36me2 modification resisted nucleosome clearing, suggesting the modification stabilizes the nucleosomes.

The Set2 family of methyltransferases are generally responsible for the di- and trimethylation of H3k36me2 (62, 86). In humans, Smyd2, a SET/MYND domain containing histone H3K36 specific methyltransferase, interacts with Sin3A/HDAC to deacetylate nucleosomes in the 3' end of expressed genes, where it seems to function as a restraint on cell proliferation(12). In an interesting new twist, H3K36 acetylation is also conserved from yeast to mammals, and is present in promoters of RNA polymerase II transcribed genes(85). In S. cerevisiae, the chromodomain protein Eaf3 recognizes the H3K36me2 modification, and thereby targets the RpdS complex behind the elongating RNA polymerase II where it deacetylates the newly reassembled nucleosomes (13).

As might be suggested by the evolutionary stability of both the sequence and the acetylation state of histone H4 residues (38), a simple H4 code was recently proposed for *S. cerevisiae* (22). By specifically mutating histone H4 tail residues K5, K8, K12 and K16 to arginine (constitutively unacetylated), and looking at their global expression patterns in all combinations, most of the mutations were seen to cause cumulative transcriptional changes. However, H4K16R expression pattern clustered better with the *sir* mutants, indicating that it had a special code value. In a recent study, homogeneously chemically acetylated H4K16 makes compaction of the chromatin into

30nm fibres impossible, as it interferes with cross fibre interactions, and inhibits ACF mononucleosome mobilization (120).

Chromatin is the underpinning of all DNA based life, and essentially gates the production of every protein a cell makes. Therefore, increasing complexity in the modifications decorating nucleosomes may indeed be one of the keys to the development of complex multi-cellular organisms. In Garcia et al, they remark on the expansion of the nucleosome modification recognition and establishment components with advancing complexity in the organism (38). This is a fascinating way of explaining how the same basic core nucleosome can be modified in ever increasingly complex ways to allow increasingly complex modulation of gene regulation.

3 HISTONE DEACETYLASES

The most abundant post-translational modification of chromatin is the reversible acetylation of core histones. The levels of histone modification are the result of a continuous process of acetylation by histone acetyltransferases (HATs) and deacetylation by Histone deacetylases (HDACs). Whereas HATs catalyze the transfer of an acetyl moiety from acetyl-coA to the ε-amino group of lysine residues, HDACs catalyze removal of the acetyl group, thereby reconstituting the net positive charge of the histone.

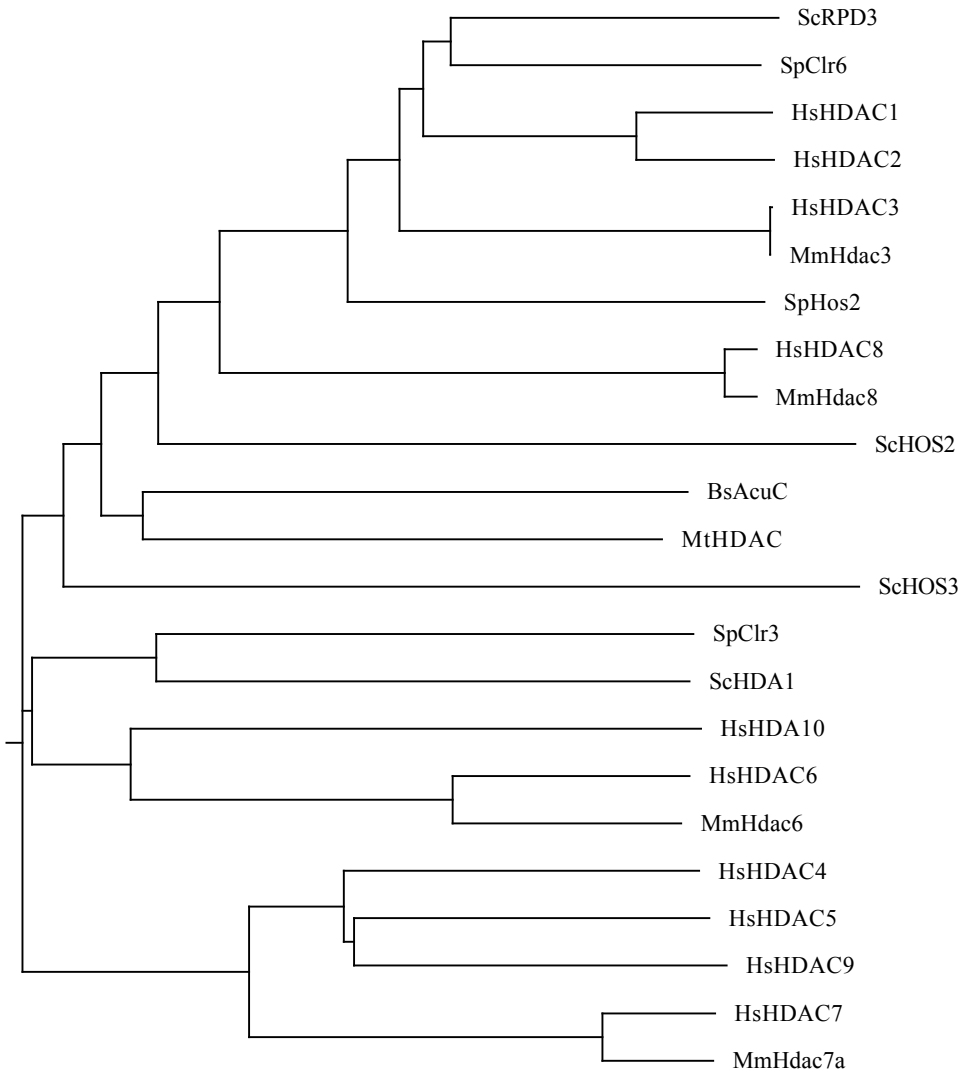

Figure 3. Amino acid sequences of human (Hs), mouse (Mm), *S. pombe* (Sp), and *S. cerevisiae* (Sc) were aligned using the clustal analysis in MacVector. *Bacillus subtilis* AcuC and an HDAC from the archaebacteria *Methanosaeta thermophila* PT were used to found the tree.

The deacetylase family of enzymes is ancient, as they are highly related to the acetoin utilization proteins and amidohydrolases from archaeal and eubacterial. (74), and have been found in all eukaryotes.

The first histone deacetylyase was isolated from human Jurkat T cells based on its ability to bind the histone deacetyase inhibitor trapoxin (130). Sequence analysis showed it to be highly related to the yeast co-repressor protein RPD3. This was followed rapidly by the isolation of HDAC activities from yeast based on their abilities to bind TSA (trichostatin A) (111). Not unexpectedly, the responsible enzymes were identified as RPD3 and HDA1 by peptide sequencing.

Histone deacetylases can be placed in three different phylogenic classes that predict their function and localization fairly well (Figure 3 and Table 1). The founding member of Class I HDACs is RPD3, and members of this class have a very high region of homology over about 300 amino acids. Class I enzymes can be described as sensitive to TSA, trapoxin and suberoylanilide hydroxamic acid. They tend to be nuclear where they associate tightly with Sin3 or with N-CoR for proper targeting to chromatin targets (19). Class I HDACs are generally ubiquitous in multicellular organisms.

Class II histone deacetylases have a similar enzymatic pocket and are therefore also sensitive to TSA, trapoxin and suberoylanilide hydroxamic acid. However, they tend to be normally localized to the cytoplasm until they are induced to enter the nucleus. In mammals, class II HDACs also tend to have a tissue specific or developmentally determined expression pattern (79, 148)

3.1 CLASS I HISTONE DEACETYLASES

The founding member of Class I HDACs is RPD3, and members of this class have a very high region of homology over about 300 amino acids. Class I enzymes can be described as sensitive to TSA, trapoxin and suberoylanilide hydroxamic acid. They tend to be nuclear and are generally ubiquitous in multicellular organisms. Association with Sin3 or with N-CoR is necessary both for the for proper targeting to chromatin targets and for their enzymatic activity (19). Only HDAC1 and -2 have been shown to interact with Sin3, and their association with the complex is regulated by phosphorylation. Both Hos2 and Clr6 are class I histone deacetylases.

Clr6 is most homologous to the budding yeast histone deacetylase RPD3. Clr6 was first isolated by its ability enhance the silencing defects in *clr1*. *clr6*+ is an essential gene, and is required for repression of polII genes placed within the centromere, the telomeres and the mating type region (39). In addition, the temperature sensitive mutant *clr6-1* has an elevated rate of chromosome mis-segregation. Clr6 has been purified in a complex with Pst1 and Pst2, and more recently with Pst3 (91, 124).

The second class I histone deacetlase in *S. pombe*, Hos2, was originally named Hda1 (98) and Phd1 (64). It was renamed because of its functional and sequence homology to ScHos2. *hos2Δ* cells experience enhanced gene repression of marker genes placed

within centromeres, telomeres, and the mating type loci. This repression could be alleviated by addition of the histone deacetylase poison Trichostatin A (98).

3.2 CLASS II HISTONE DEACETYLASES

Class II histone deacetylases have a similar enzymatic pocket to their Class I cousins and are therefore also sensitive to TSA, trapoxin and suberoylanilide hydroxamic acid. However, they are usually longer, and tend to be localized to the cytoplasm in the absence of signals that will enable them to enter the nucleus. In mammals, class II HDACs tend to have a tissue specific or developmentally determined expression pattern (79, 148)

The founding member of this class of histone deacetylases is HDA1 from *S. cerevisiae*, and was isolated in the initial biochemical screen based on TSA binding (111). ScHda1 is targeted by Hda2 and Hda3(155) as well as Tup1(156)

Table 1. Classification of HDACs from *Saccharomyces cerevisiae* (Sc), *Schizosaccharomyces pombe* (Sp), *human* (Hs), *mouse* (Mm) and *Drosophila melanogaster* (Dm). Table adapted from (79).

Class	Members	Properties
I. ScRpd3 like	ScRPD3, Sc, Hs, Mm, DmHDAC1, SpClr6; Sc, Hs, Mm, Dm HDAC2 Sc, Hs, Mm, Dm HDAC3 HsHDAC8 HsHDAC11 Sc, SpHOS2 ScHOS3	TSA. SAHA, TPX sensitive; N-terminal deacetylase domain; nuclear localization; associated with Sin3 and N-CoR
II. ScHda1-like	ScHDA1 SpClr3; HsHDAC4[a]; HsHDAC5[a]; HsHDAC6[b]; HsHDAC7[a]; HsHDAC9[a]; HsHDAC10[b]; MmHDA1; Mm, DmHDA2; ScHOS1	TSA. SAHA, TPX sensitive; C-terminal deacetylase domain; nucleocytoplasmic shuttling; does not interact with Sin3. [a] sub-class with extended homologous N-terminal domain [b] sub-class with two HDAC domains
III. ScSir2-like	Sc, SpSIR2, ScHST1, HsSIRT1 Sc, SpHST2, HsSIRT2, HsSIRT3 ScHST3, Sc, SpHST4 HsSIRT4; HsSIR2T5; HsSIRT6; HsSIRT7	NAD-dependent histone deacetylases

In humans, the class two enzymes have been further broken down into two sub classes. Class IIa enzymes HDAC4, -5, -7, -9 have highly conserved c-terminal catalytic domains with novel N-terminal domain of homology. HDAC4, -5, -9 are expressed specifically in the heart, skeletal muscle and brain (139). HDAC7 is expressed in thymocytes (reviewed in (20)). Class IIa HDACs are targeted by in several ways to chromatin. For instance, HDAC4 and -5 interact directly with CtBP1 (E1A C-terminal binding protein) (reviewed in (163)). The key to nucleocytoplasmic shuttling of these enzymes depends on14-3-3 proteins which bind phosporylated residues and sequester the HDACs to the cytoplasm (82)

Class IIb enzymes are also primarily cytoplasmic and can also translocate to the nucleus. HDAC6 has been associated with cell cycle arrest (137), and is predicted to deacetylate non-histone proteins before they are ubiquinated (48, 117).

S. pombe has one class II histone deacetylase. Clr3 was originally isolated in a screen to identify mutants which could not maintain silencing within the K region of the mating type locus (32). Early characterizations of *clr3-E36*, and later *clr3Δ* indicated a role for Clr3 in centromeric, telomeric, and rDNA silencing (10, 39). Although Clr3 is in the cytoplasm, it is primarily nuclear, inhabiting both the chromatin and nucleolar portions of the nucleus, with a high concentration at the outer periphery of the nucleus near the nuclear membrane (10).

It is interesting to note that in 2001 structural biologists had classified both Hos2 and HsHDAC8 as a type II enzymes (79). This is particularly interesting because SpHos2, ScHos2 and HDAC8 are primarily cytoplasmic. What is more there is no evidence that ScHos2 interacts with Sin3, and unlike the other class I HDACs in S. cerevisiae. In addition, SpHos2 has an in vitro activity that does not require interaction with a corepressor (64).

3.3 CLASS III HISTONE DEACETYLASES (SIRTUINS)

Class III HDACs, often referred to as Sirtuins, consist of a more divergent group of Nicotinamide adenine dinucleotide (NAD) dependent enzymes (reviewed in (11)). The founding sirtuin, ScSir2, was isolated in a genetic screen for silencing at mating type (109). Sir2 is required for silencing of all heterochrmomatically silenced regions in *S. pombe* (36, 156). Hst4 is another *S. pombe* sirtuin, and has been implicated in telomere position effects, radiation damage response and chromosome segregation (37, 118) just like its *S. cerevisiae* homolog Hst2 (102).

4 SIN3 CO-REGULATORS

Histone deacetylases do not bind DNA, but are rather targeted within a complex, such as the Sin3/HDAC complex. Sin3 complexes have mostly been characterized in conjuction with class I histone deacetylases. SIN3 was isolated in several genetic screens. SIN3 gets its name from the SWI-independent screen *(125)* although it was identified in a parallel screen as SDI1 *(93)*. Both screens were designed to identify genes necessary in the highly regulated mating type switching in *S. cerevisiae.*

Mating type switching is a tightly regulated gene conversion event catalyzed by HO endonuclease. Switching occurs only in late G1 in mother cells, but mutations to SIN3 did not affect cell cycle regulation of *HO*. On the other hand, *sin3* could depress *HO* in daughter cells, bypassing the need for *SWI5* to activate *HO* transcription specifically in mother cells *(93, 125)*. Interestingly, Nasmyth and colleagues also isolated *RPD3* (*SDI2*) and concluded that the two were working cooperatively to derepress *HO*.

SIN3 was also isolated in a screen for unscheduled meiotic gene expression (*UME4*),

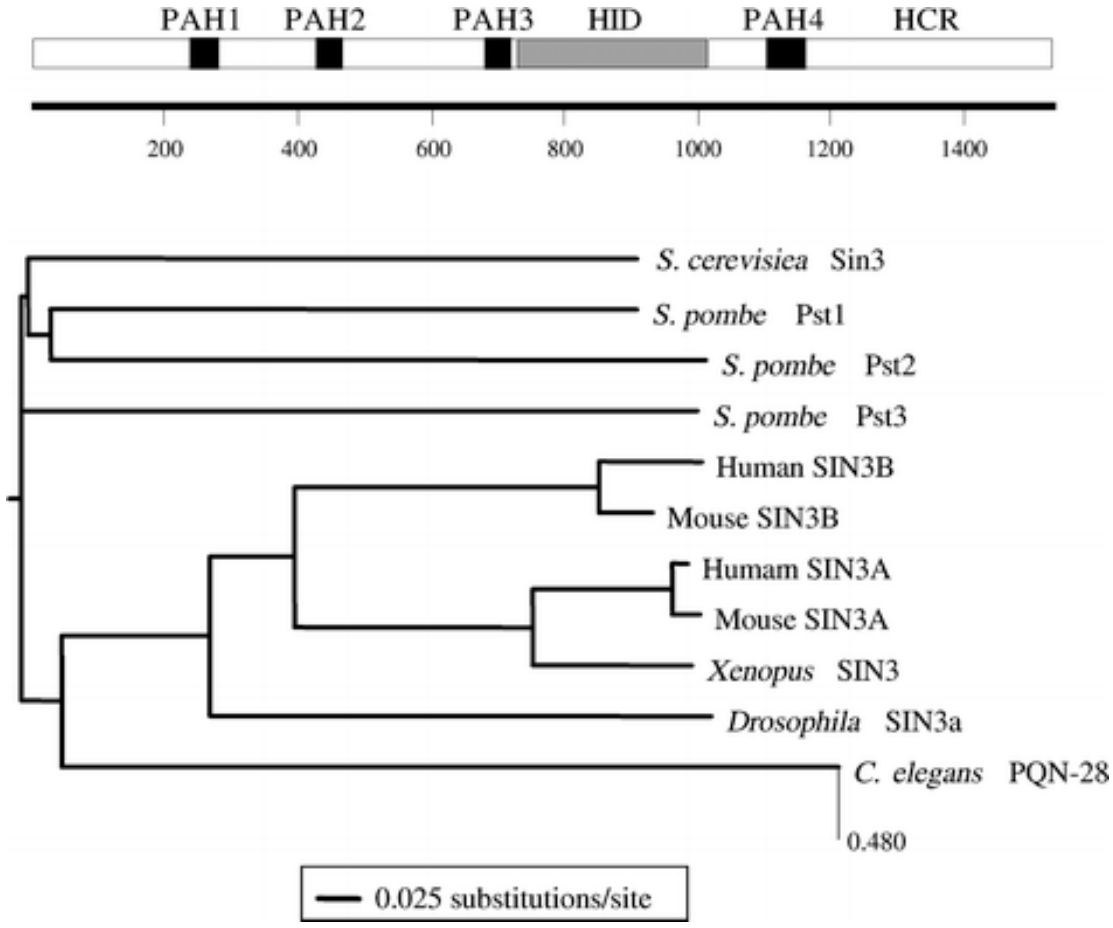

Figure 4. Sin3 phylogenetic tree. The duplications of the Sin3 molecules in S. pombe appear to be ancient. The duplication in mammalians seems to also be ancient. However, the multiple Sin3s seemed to have evolve independently.

along with the transcription factor *UME6,* which often targets the SIN3/RPD3 complex, and *UME1,* which was later found to be part of the SIN3/RPD3 complex (127). *SIN3* was identified in a screen to detect proteins with reduced potassium dependence (*RPD1*) as a negative regulator of low affinity K+ transporter *TRK2.* Again *RPD3* was isolated in the same screen (141). SIN3 (*SDS16*) and RPD3 (SDS12) were isolated together with a constitutive member of the SIN3/RPD3 complex SDS3 a third time in a screen designed to reveal suppressors of *rap1* induced *HMR* derepression (135)

SIN3 homologs have been identified in most eukaryotes, usually interacting with class I histone deacetylases. *S. cerevisiae* has only one SIN3 molecule, whereas *S. pombe* has three (Figure 4). Mammals have been shown to encode two SIN3 homologs: SIN3A SIN3B, in mouse B has a shorter tail (43). Evidence also exists that splice variants of both SIN3A and SIN3B exist in both humans and mice (1, 160). Interestingly, Human Sin3A is more related to mouse Sin3A than to human Sin3B, suggesting duplication occurred before speciation (122).

SIN3 is a large acidic protein, predicted to be 174.9 kDa in yeast. The most conserved domains of SIN3 are the four paired amphipathic α-helices (PAH1-PAH4) (151) (Figure 4.1.1). The similarity of the PAH domain sequences to the myc helix-loop-helix protein dimerization domains led researchers to correctly predict that these domains would be involved in protein::protein interactions (4, 43). Another highly conserved region located between PAH3 and PAH4 was shown to be essential for full repression activity of RPD3 in tissue culture (152). This region was later identified as the HDAC interaction domain (HID). The fifth highly conserved domain PAH4 is required for full repression by Sin3, but its precise function remains uncharacterized. An identity of only 9% with the other PAH domains and the number of divergent substitutions imply that PAH4 will have a different binding pocket than the other domains (133). Using NMR, investigators have been able to predict that PAH1, -2 and -3 will form pre-folded binding modules in full-length Sin3 to act as folding templates for the interaction domains of target peptides. Different thermodynamic stability of PAH domains mSin3A and mSin3B may be related to functional differences in their protein interaction potential(133) (Figure 4.1.2).

4.1 CORE SIN3/HDAC COMPLEX

In fact, most of the characterized functions of Sin3 are related to its ability to interact with many different kinds of proteins and thereby providing a platform to coordinate the localization of specific combinations of DNA binding factors and chromatin modifying enzymes. At the core of the Sin3/HDAC complex are approximately seven subunits: Sin3, HDAC1, HDAC2, RbAp46, RbAp48, SAP30, SAP18 and possibly SDS3 (46, 68, 169). However, the broad elution pattern of mSin3 complex from size exclusion columns suggest there are multiple mSin3 complexes (169).

HDACs provide enzymatic activity to the Sin3/HDAC complex. The homology of SIN3/HDAC complex to the already characterized SIN3/RPD3 proteins gave budding yeast researchers a head start on understanding how histone deacetylation would effect gene transcription. For instance, Ume6 controlled genes had elevated histone H4K5 acetylation in *rpd3* strains, but interestingly H4K5 and H4K12 acetylation levels were

raised in *sin3* (112). In addition, the well characterized URS1 element from the IME2 promoter could be shown to be hyperacetylated at histones H4K5, H4K12, and H4K16 in both *rpd3* and *sin3* mutants (56). However, the simple presence of Sin3 and HDAC1 was not always sufficient to induce repression. In *Xenopus* oocytes, Sin3 and HDAC1 were constitutively present at signal responsive promoters even if hypoacetylation and gene repression only occurred after induction (76).

The small WD-repeat proteins RbAp48 and RbAp46 were also identified as part of the core complex, although they were originally isolated for ability to bind immobilized fragments of the transcription factor Rb (retinoblastoma) (106, 107). these proteins have been shown to interact directly with histones H4 and H2A (140) and thus are predicted to help stabilize the interaction between Sin3/HDAC complex and histone H4. WD repeat proteins are conserved members of the Sin3/HDAC core as homologs have been identified in the other Sin3/HDAC complexes isolated *Drosophila* (132) and *S. pombe* (91, 124).

SAP30 interacts with Sin3 via PAH3 and HDAC1 (170). NMR structural analysis of the SAP30 region bound to the transcription factor Su(fu) was shown to be structurally similar to cryptic ubiquitin-like beta-grasp domain used by several other proteins in the assembly of larger protein complexes (63, 80). Thus, Sap30 is predicted to stabilize Sin3/HDAC complex interactions with peripheral protein interactions. At rDNA loci in humans there are two remodeling complexes, NoRC and WICH, that oppose each other's function (75, 101). NoRC has been demonstrated to recruit Sin3/HDAC to mediate repression (171). Interestingly, some evidence exists that SAP30 is required to recruit the Sin3/HDAC complex to the nucleolus, where it regulates transcription of rDNA genes (144). It will be interesting to see if SAP30 is mediating an interaction between the HDAC and NoRC complexes, and if the Pst3 complex will be targeted in a similar way.

SAP18 is conserved from yeast to humans and ubiquitously expressed in mouse tissues where it has been shown to interact with SIN3 and HDAC1, although possibly not HDAC2 (169). SAP18 has been shown to bind several transcription factors as well (14, 34, 172). For instance SAP18 interacts with GAGA to regulate the *iab-6* element of the bithorax complex during *Drosophila* development (34). Thus SAP18 apears to function as an adaptor between transcription factors and Sin3/HDAC complex (14). Interestingly, SAP18 has a structural homolog in *S. pombe* (SPCC126.13c), while budding yeast does not. This may be in part because the budding yeast SIN3/RPD3 complex is usually guided by transcription factor Ume6.

It has been postulated that SDS3, although not in original purifications, may be part of the core complex. In yeast, SDS3 RPD3 and SIN3 affect the expression of the same set of genes (135) and it seems to be present in the Sin3/RPD3 complex (26, 73).

It is important to note that although these eight proteins make up the core complex not all of them are always found in the complex. In the original purifications, the Rb associated proteins did not appear to be present in stochiometric amounts (169). At least on functional complex has been purified containing HDAC2 but not HDAC1(121). The SAP proteins were not present in some other purifications (53, 95).

This could be reflecting the variability that is intrinsic to biochemical purifications, or a signal that the functional Sin3/HDAC complex is anything other than stable.

4.2 TARGETING OF SIN3

As mentioned earlier, Sin3 has no intrinsic DNA binding activity. Therefore Sin3 must be directed to its targets by binding with transcription factors (Table 2). Mad1 was the first transcription factor found to interact with Sin3 (4). Mad/Max as well as the other Mad homo- and heterodimers are natural antagonists to Myc/Max, and thus promote withdrawal from the cell cycle and terminal differentiation (reviewed in (7)). The antagonist function of Mad and Mnt has been shown to require interaction with Sin3 (28, 49, 59). In fact, all of Max heterodimer partners, except Mga, have a conserved Sin3 interaction domain (SID) that allow them to recruit HDAC-dependent repression to their target genes (28, 45).

Because Sin3 cannot itself bind DNA, the interaction between transcription factor and Sin3 is often a target for regulation. In the case of Ume6, a positive and negative regulator of meiotic gene transcription in *S. cerevisiae*, a third protein Ime1 is able to interfere with the Sin3/Ume6 interaction under meiosis-stimulating conditions thereby relieving the HDAC-dependent repression (153). Phosphorylation can also be used to alter the interaction between transcription factors and Sin3. In some cases phosphorylation at or near the SID can alter the binding affinity between Sin3 and the transcription factor (33, 50). Sin3 can even be used to fine tune the repressor activity of a transcription factor as it does for Mxi. Mxi has two splice variants, one of which is missing the SID but still maintains a low level of repressor activity. By balancing the amount of full length Mxi that is a potent repressor, with the weaker truncated Mxi the cell can fine-tune the degree to which a target gene will be repressed (115). Interestingly, this kind of competition has also been characterized for truncated versions of Sin3 itself, which can be bound by transcription factors, but lack the HID (1, 160). Thus the interaction-based targeting of Sin3 enables the complex to be regulated at many more levels than an intrinsic DNA binding activity would allow.

Table 2. Proteins interacting with Sin3 have a wide range of functions. For those characterized, most commonly interact with an α-helix in PAH pocket. Adapted from (122). NA indicates not addressed.

Factor	Functions	SID	Sin3 Interface	Ref.
SMRTER	*Drosophila* corepressor for nuclear hormone receptor EcR	DALA α-helix motif	PAH1	(131)
Opi1	Transcriptional repressor for phospholipid biosynthesis in *S. cerevisiae*.	Amphipathic α-helix (53-79 AA)	PAH1	(149)
HCF-1	Regulator of cell proliferation in metazoans. Links histone methyltransferases to Sin3/HDAC complex.	Basic domain	PAH1	(157)
Pf1	Transcription factor that links Sin3/HDAC complex with groucho/TLE complex to globally regulate gene expression	Pf1SID1: amphipathic α-helix; A[AV]X[VA][LV] motif	PAH1; PAH2	(72, 164)

N-CoR	Corepressor for nuclear hormone receptors	DALA α-helix motif	PAH1; PAH3-HID, SAP30	(1, 47)
LAZ3/ BCL-6	DNA binding transcription factor associated with non-Hodgkin lymphomas	Central residues: 191-383	N-terminal	(21)
Mad family	Max heterodimers. Antagonize Myc/Max activation. Involved in mammalian cell proliferation and differentiation. Family includes: Mxi, Mnt.	A[AV]X[VA][LV] motif: 13-20AA α-helix	PAH2	(4, 49, 115)
Ume6	Transcription factor that positively and negatively regulates meiosis-specific genes in *S. cerevisiae.*	A[AV]X[VA][LV] motif: 13-20AA α-helix	PAH2	(55, 72, 153)
Stb1-5	Isolated in a two-hybrid screen as Sin Three binding proteins.	NA	PAH2	(60)
REST/NRSF	Repression of neuronal genes in non-neuronal tissues. Also functions with Sin3 in yeast.	A[AV]X[VA][LV] α-helix motif N-terminus	PAH2	(41, 72, 110)
Sp1-like transcription factors	Family of repressors that regulate mammalian cell homeostasis: TIEG2, TIEG1, BTEB1, BTEB3, BTEB4	SID-like: amphipathic α-helix	PAH2	(33, 167)
TIS7	Transcription factor regulating epithelial cell polarity in mouse mammary glands	N-terminal 140 AA	PAH2 Sin3B	(143)
P53	Tumor suppressor governing apoptosis. Most commonly mutated gene in human cancers.	Proline rich domain AA 40-160	Between PAH2 and PAH3	(87, 174)
AML1	Acute myeloid leukemia 1 transcription factor regulates hematopoietic genes	Residues 172-237	PAH3	(78)
Elk-1	MAPK-inducible transcription factor involved in the up-regulation of immediate-early genes in response to growth factor, and their subsequent repression.	N-terminal ETS DNA binding domain (phosphorylated)	PAH3	(161)
MeCP2	Methy-CpG-binding protein involved in the long-term repression of genes during mammalian development.	Central region (also binds HDAC1 & HDAC2).	PAH3-HID; C-terminal end Sin3A only	(92)
OGT	An O-GlcNAc transferase	TPR multiple amphipathic α-helices	PAH4	(162)
Alien	Corepressor for nuclear hormone receptors	N-terminal 128 AA	HID; HCR	(84)
MNFα, MNFβ	Winged-helix/forkhead transcription factors governing terminal differentiation of muscle cells	N-terminal domain	Sin3B	(160)
NRL	Neuronal retinal leucine zipper transcription factor	α-helix 125-150AA	PAH2	(72)
SMRT	Corepressor for nuclear hormone receptors	Extensive (Sin3A only)		(89)
Hesx-1	Transcription factor required, in combination with TLE1, to prevent induction of multiple pituitary glands from the oral ectoderm.	homeodomain	NA	(17)
HERP1, HERP2	Regulates cell fates in metazoans by regulating the Notch signaling pathway.	BHLH domain	NA	(51)
ESET	H3 specific methyltransferase.	Tudor domain	NA	(158)
SAP25	Putative part of core complex	LXXLL 125-186AA	PAH1	(119)
Alp13, Eaf3	Chromodomain proteins that bind H3K36me following transcriptional elongation	NA	Pst3	(13, 62, 124)
NoRC	SNF2h-containing nucleolar chromatin	Immunopurifies with the core		(171)

	remodeling complex	complex	
Smyd2	SET/MYND H3K36 methyltransferase. Unknown function, embryonal specific to heart and hypothalamus, linked to cell proliferation	NA	(12)
MORF4, MRGX, MRG15	Mortality factor family of transcription factors contributing to cellular senescence.	NA	(165)
CHES1/ FOXN3	Transcription factors for suppression of DNA damage checkpoints	NA	(116)
RBP1	Corepressor for tumor suppressor Rb; functions in senescence, development and cell cycle exit.	R2 repression domain interacts directly with SAP30 not Sin3; possibly PAH2 via LXXLL	(69) (71)
p33ING1b	Transcription factor that inhibits cell growth in cooperation with p53	Unique N-terminal domain binds SAP30 not Sin3	(67)
Suv39H1	Histone methyltransferase	Binds core components: HDAC1, HDAC2, RbAp46, RbAp48 (Sin3 not tested)	(136)

4.3 SIN3 IN GENE ACTIVATION

Although a few years ago there appeared to be a general consensus in the scientific community that Sin3 is primarily repressive, the growing body of evidence linking Sin3 transcription and activation has begun to shift this preconception. Genetic evidence that Sin3 would be involved in positive gene regulation existed before the Sin3/HDAC complex was characterized. In 1991, a detailed examination of inducible genes indicated that Sin3 was required not only for the maximal repression of those genes, but for their full activation as well (142). And in 1992, Sin3 was isolated in a screen designed to find genes required for the trans-activation of *STA1* (166). More recently, detailed molecular investigation revealed that Hog1 could not induce osmoresponsive genes in the absence of Sin3 or RPD3 (18).

Genomic studies have supported a role for Sin3 in gene activation as well. In yeast, 198 genes were found to be down regulated in *sin3*(9). That is twice as many as they found were up-regulated. In *Drosophila*, the number of down-regulated genes were more meagre, only 35, but some of those genes encoded proteins involved in cell cycle control that could explain previously seen phenotypes (104). In S. pombe, both *pst1-1* and *pst3D* cause a large number of genes to be expressed at lower levels than in wt (unpublished results, (124)).

This can be viewed at another angle as well. Global histone acetylation maps published by Kurdistani and colleagues (66) demonstrated that hypo-acetylation did not always correlate to low gene expression, and like-wise hyper-acetylation did not always correlate with high gene expression. In fact, the acetylation of H4K16, H4K8, and H4K12 were anti-correlated with gene expression. Acetylation maps made in *S. pombe* similarly indicated H4K12 and H4K5 acetylation levels were also anti-correlated with gene expression in both ORF and IGR (154). The conserved effect on histone H4 is particularly interesting in light of the observation that histone H4 modifications are more evolutionarily stable (38).

The positive role in gene transcription may be the result of the coding region functions of the Rpd3S complex. This complex contains Rpd3, Sin3, Ume1, Ume6, Rco1 and

chromodomain protein Eaf3. Eaf3 binds H3K36 methylated by Set2 during transcriptional elongation. The role of this complex seems to be the removal of transcription-specific acetylation marks to stabilize the nucleosome so that spurious intergenic transcription does not initiate (13). It therefore seems significant that the positive gene regulation of Pst1 and Pst3 correlates with ORF binding, and that Pst3 interacts with Alp13, the fission yeast homolog of Eaf3 (unpublished results, (124)).

4.4 SIN3/HDAC ACTIVITY BEYOND HISTONE DEACETYLATION

The core complex interacts with Sin3 in and around the HID domain. Transcription factors interact most frequently through PAH1, although PAH2 and PAH3 interactions have been characterized. However, Sin3 also provides a scaffold for many other enzymatic activities associated with Sin3/HDAC complex.

There are several examples of nucleosome remodeling enzymes associating with Sin3/HDAC complex. Affinity purification of Swi/Snf from human tissue culture using Flag-Brg1 or Flag-Brm resulted in two slightly different complexes(121). Flag-Brg1 did not contain HDAC1 could open whole nucleosome in vitro to DNAse digestion, whereas Flag-Brm only disrupted nucleosomes at their edges, and was much more active on nucleosome arrays than mono-nucleosomes. In addition, the SNF2h-containing nucleolar chromatin remodeling complex NoRC could be shown to interact with Sin3 and HDAC1 both using co-sedimentation and Co-immunoprecipitation (171). NoRC interacts with HDAC1 and Sin3 via the subunit TIP5 (TTF-I interacting protein) to silence rDNA by sliding nucleosomes in an ATP and H4 tail dependent fashion (128). Considering Pst3 interacts with Snf59, a subunit of the RSC remodeling complex, it will be interesting to see if Pst3 is also recruited to chromosome regions by remodeling complexes (124).

Sin3/HDAC can also interact with histone methyltransferases. In mouse, the H3K9me2 to specific H3K9me3 methyltransferase ESET is required for the earliest stages in mouse embryonic development (23). It interacts with HDAC1, HDAC2, Sin3A/B via an N-terminal tudor domainand has a methyl-CpG binding domain (159). ESET can repress transcription independently of its histone methyltransferase activity, but requires SIN3 to bind to its target genes (150).

Smyd2, a split SET/MYND domain-containing histone H3K36 specific methyltransferase, can be co-immunoprecipitated with Sin3 in mammalian cells. Interestingly, over-expression of Smyd2 caused reduced growth rates in NIH3T3 cells (12). Smyd2 is predicted to have a similar role to Eaf2 from the Rpd3S complex, as H3K36 methylation is tightly associated with actively transcribed genes in coding regions (5, 108).

Thus, by exchanging its interaction partners, Sin3 can be involved in gene repression and gene activation. The system is flexible enough to be conserved through evolution, where it has been employed to modulate everything from cell stress response pathway in unicellular eukaryotes to embryonic development and cell proliferation in mammals.

5 SCHIZOSACCHAROMYCES POMBE AS A MODEL ORGANISM

The fission yeast, *Schizosaccharomyces pombe*, has been used as the model organism for this study. It is a unicellular ascomycete that is estimated to have diverged from *Saccharomyces cerevisiae* about 1,144 million years ago. Since animals plants and fungi are predicted to have diverged about 1,576 million years ago, the two yeast model organisms and humans are nearly evolutionarily equidistant from one another (Chapter 29. (27)). Thus by identifying mechanisms conserved between both yeast, and any of the multicellular model organisms we are provided with insight into the fundamental mechanisms of cellular function likely to be true in humans as well.

Among the advantages for choosing fission yeast as a model organism are its well defined classical and molecular genetics, in addition to its well characterized chromosome cell biology and cell cycle. As with most model organisms, the simplicity of the system makes it easier to design controlled experiments to specifically test a hypothesis. That S. pombe naturally exists most predominantly as a haploid, and its genome has been entirely sequenced, only makes the genetics easier. *S. pombe* has a few specific advantages when studying heterochromatin. First its centromeres are very structurally similar to their human counterparts, and the other heterochromatic regions have been well characterized. Second, the heterochromatic silencing mechanisms uncovered so far have been highly analogous to those found in higher organisms. And third, unlike tissue culture cells that often have aneuploidy or other markers of heterochromatic disturbance as a result of their unnatural immortalization, wt S. pombe is generally unstressed and heterochromatically stable.

6 RESULTS AND DISCUSSION

6.1 PAPER I: TRANSIENT INHIBITION OF HISTONE DEACETYLASE ACTIVITY OVERCOMES SILENCING IN THE MATING-TYPE REGION IN FISSION YEAST

In this paper we investigated the effects of TSA on the silent mating-type locus. We found that TSA could partially de-repress a marker gene inserted 150 base pairs distal from the silent *mat3-M* locus (1999). This confirmed that histone deacetylation was involved in mating type silencing. At the time, only Hos2 (Hda1) had been characterized, and the genome sequencing was not yet complete. In the discussion, we argue that another TSA sensitive HDAC than Hos2 must be responsible for the silencing at the mating type locus.

6.2 PAPER II: DICER IS REQUIRED FOR CHROMOSOME SEGREGATION AND GENE SILENCING IN FISSION YEAST CELLS

Dcr1 is the *S. pombe* homolog of human Dicer, a nuclease that cleaves dsRNA into small interfering RNAs that can cause specific gene silencing in a mechanism known as RNA interference or RNAi. As predicted by its homology to Dicer in other species, we found that Dcr1 was able to degrade double-stranded RNA into approximately 23 nucleotide fragments *in vitro*, and *dcr1*Δ cells were partially rescued by expression of human Dicer, indicating evolutionarily conserved functions. Expression profiling demonstrated that *dcr1+* was required for silencing of two genes containing a conserved motif. Deletion of *dcr1+* caused slow growth, sensitivity to thiabendazole, lagging chromosomes during anaphase, and abrogated silencing of centromeric repeats.

This paper was one of three papers published within the year showing that Dicer had a conserved role in heterochromatic silencing and possibly gene regulation (42, 146). The number of genes estimated to be regulated by Dcr1 in this paper seems to be underestimated. Hansen et al. demonstrated that dcr1 mutants up-regulated approximately 76 genes (44).

6.3 PAPER III: A NEW ROLE FOR THE TRANSCRIPTIONAL COREPRESSOR SIN3: REGULATION OF CENTROMERES.

In this study, we have characterized *S. pombe* Sin3 corepressors (Pst1p and Pst2p) to investigate whether SIN3-HDAC is required in the regulation of centromeres. We show that only *pst1-1* but not *pst2Δ* cells displayed chromosome segregation defects and thiabendazole sensitivity. *pst1-1* cells showed reduced centromeric silencing, increased histone acetylation in centromeric chromatin, and defective centromeric sister chromatid cohesion. The HDAC Clr6p and Pst1p coimmunoprecipitated, and partially colocalized. Clr6 like Pst1 is an essential gene, mutants of which display increased chromosome mis-segregation and abolished hypoacetylation at centromeres. Pst1 could be seen decorating the centromeres in binucleate cells predicted to be in S-phase. These data are consistent with a model in which Pst1p-Clr6p temporally associate with centromeres to carry out the initial deacetylation of newly laid nucleosomes that would

be necessary for subsequent steps in heterochromatin formation. Remarkably, unlike *clr6-1*, *pst1-1* does not cause the bulk histones to become hyperacetylated, indicating that their interaction is not exclusive, as might be expected from Sin3/Rpd3.

6.4 PAPER IV: FUNCTIONAL DIVERGENCE BETWEEN HISTONE DEACETYLASES IN FISSION YEAST BY DISTINCT CELLULAR LOCALIZATION AND IN VIVO SPECIFICITY

This paper focuses on understanding the division of labor for three of the *S. pombe* histone deacetylases *hda1, clr3*and *clr6*. Genetic analysis showed that strains carrying mutations in the different histone deacetylases display strikingly different phenotypes when assayed for viability, chromosome loss, and silencing. The localization of the three hdacs is also distinct. Both Clr3-myc and Clr6-HA had mottled nuclear localization patterns, with Clr6 limited to the chromatin, while Clr3 could also be found in the nucleolus. Clr3 was also strongly present in the space nearest the nuclear envelope. In contrast, Hos2-HA (Hda1-HA) shows cytoplasmic staining in healthy growing cells. Attempts to co-immunoprecipitate any of the HDACs together, indicating that they are not tightly associated. In addition, each of the HDACs was shown to have a different impact on global histone acetylation levels, with Clr6 having the broadest effect and Clr3 having a specificity for H3K14ac

6.5 PAPER V: GENOMEWIDE ANALYSIS OF NUCLEOSOME DENSITY HISTONE ACETYLATION AND HDAC FUNCTION IN FISSION YEAST

We have conducted a genomewide investigation into the enzymatic specificity, expression profiles, and binding locations of four histone deacetylases (HDACs), representing the three different phylogenetic classes in fission yeast (Schizosaccharomyces pombe). By directly comparing nucleosome density, histone acetylation patterns and HDAC binding in both intergenic and coding regions with gene expression profiles, we found that Sir2 (class III) and Hos2 (class I) have a role in preventing histone loss; Clr6 (class I) is the principal enzyme in promoter-localized repression. Hos2 has an unexpected role in promoting high expression of growth-related genes by deacetylating H4K16Ac in their open reading frames. Clr3 (class II) acts cooperatively with Sir2 throughout the genome, including the silent regions: rDNA, centromeres, mat2/3 and telomeres. The most significant acetylation sites are H3K14Ac for Clr3 and H3K9Ac for Sir2 at their genomic targets. Clr3 also affects subtelomeric regions which contain clustered stress- and meiosis-induced genes. Thus, this combined genomic approach has uncovered different roles for fission yeast HDACs at the silent regions in repression and activation of gene expression.

6.6 PAPER VI: SIN3 HOMOLOG PST3 A FACTOR IN NULEOLAR FUNCTION

In this paper we explore Pst3, the most ancient of the Sin3 molecules, in *Schizosaccharomyces pombe*. In contrast to Pst1 (123) and Pst2 (91), Pst3 occupies the entire nuclear space, including the nucleolus. The deletion of *pst3*+ causes general genome instability including chomosome mis-segregation, gross sporulation defects, rampant anneuploidy, and distended nucleoli. Genome-wide expression analysis

indicated a role in both gene repression and gene activation. Interestingly, highly expressed genes encoding ribosomal and nucleolar proteins were positively regulated by Pst3. Genome wide binding analysis for Pst3-GFP indicated that Pst3 is bound both to intergenic and coding regions, and this binding could be correlated with expression data. What is more, proteins previously identified as part of the Clr6/Pst2 complex co-immunopurified with Pst3-TAP. Additionally, Snf59, a homolog of the RSC complex component RSC7, the Ser/Thr kinase Ssp1, and the Dead-Box helicase Dbp10 were identified as Pst3 interaction partners. Taken together this data suggests the data in this paper indicate that Pst3 has a direct role in the structure and function of the nucleolus.

7 IMPACT AND FUTURE PERSPECTIVES

Knowledge of the mechanisms that generate and perpetuate chromatin states has grown exponentially during the years I have spent as a graduate student (Figure 5). The cornerstones have been laid, the tools have been developed, and some of the supporting structure has been put in place. What remains is to understand what it all means.

Our first experiments were designed more or less as probes into the unknown. to initially observe the effects of inhibiting histone deacetylation on heterochromatin and cell function. Today, we have a basic understanding of how chromatin is organized. We also know many of the players in its assembly and modulation, and have the tools to observe their effects gene-by-gene and nucleosome-by-nucleosome. As our body of knowledge grows, it seems that heterochromatin and gene regulation are simply variations on a theme. The study of heterochromatin has given us insight into transcriptional regulation, as many of the same mechanisms are at work in both places, including transcription, nucleosome remodelling, and the balance between histone modifications. The opposite is also true: the study of transcriptional regulation is also the study of remodelling and the balance between histone modifications.

New techniques now allow us to observe global changes in everything from bulk histone modifications by mass spectrometry, to genome-wide analysis of protein binding and histone modifications. These tools will be key to understanding which of the mechanisms are the most fundamental and thus have been preserved through evolution. I believe the growing challenge now is not learning what is involved in each process, but rather how to investigate the ways in which the processes are interconnected and coordinated to allow the cell to respond to environmental challenges.

A layman might ask why we should study chromatin. Part of the answer, of course, is the standard medical reply: Chromatin is the packaging for our genetic material. By studying it, we study how the cell establishes which genes should or should not be expressed, given the specific spatial and temporal conditions of the cell. It is the key for developmental progression from completely undetermined and undifferentiated cells to fully differentiated and functional tissues. That is to say that chromatin, although possibly not determinant, plays a key role in ‘remembering’ decisions made by a cell at each step in the differentiation process by recording the ‘on’ or ‘off’ state for each gene in the chromatin itself. Many diseases, most notably cancer, often begin with a cell ‘forgetting’ its identity. Such a cell can in rare cases undermine its chromatin memory and create a new identity for itself, allowing it to grow and spread in uncharacteristic and often deadly ways.

Occasionally one is met with skepticism when trying to draw analogies between chromatin level gene regulation in yeast and human development or disease progression. However, given the fact that the cellular memory is at the level of the nucleosome, it would be foolish not to glean clues from the careful research done on gene regulation in yeast. In fact developmental decisions in higher organisms, when reduced to their simplest forms, are not very different from the decisions a yeast cell

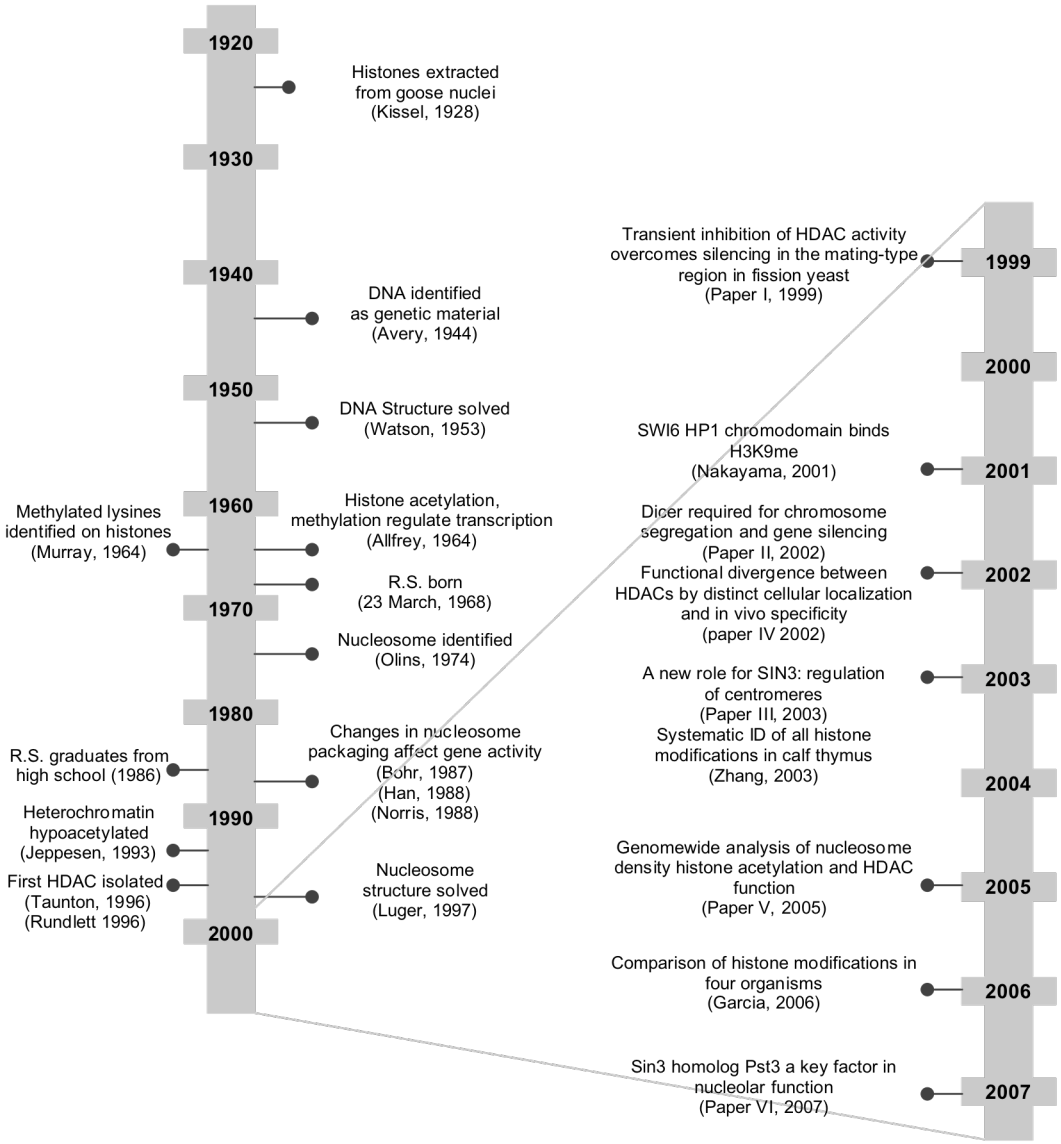

Figure 5. Timeline of major advances in chromatin research, and the author's work in the field.

makes in response to environmental signals. In both cases, one or several genes must change their chromatin structure in response to a combination of internal and external signals. The greatest differences are those requiring a change to be perpetuated even in the absence of external signals: Yeast cells can be seen as more or less totipotent, meaning they can reverse any given decision in response to new signals, while terminally differentiated cells cannot reverse the decisions made at earlier times in the developmental process. Heterochromatin is a model for terminally differentiated cells.

There is also a second, more philosophical reason to study chromatin and epigenetics. Epigenetics is in essence the study of how things can be the same and yet different. DNA and the nucleosome are essentially the same between yeast and humans. Although the actual sequence of the DNA is fundamentally different, the governing principals in the organization, and regulation are essentially identical, if more complex in the more evolved organisms. Nearly every cell in a healthy multi-cellular individual

has exactly the same DNA. However, nobody would claim a heart and an eye are the same. It is the epigenetic tool kit that allows them to be identical at their core, and yet so different in function. Is it any wonder that no two individuals, not even identical twins, are exactly the same?

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To my friends: Niklas & Lotta, Alia & Henrik, Meghan & Fred, Karin & Erik, Genni & Ingemar, & of course Monica: “It is not so much our friends’ help that helps us, as the confident knowledge that they will help us”
-Epicurus (341-270 BCE)

There are not words enough to express my gratitude for the love and support I receive daily from my husband, without whom I would have neither keel nor course. Just in the last few weeks he has had to fill the shoes of a maid, a nanny, a cook, a loyal friend, a copy editor, and a nursemaid. I love you.

I would like to dedicate this thesis to the two wonderful souls who reminded me to look at the trees, and taught me so much about living. Helene Jeanne Silverstein and Harold Edward Silverstein. I love you both to tears.

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